Oral biofilm and caries-infiltrant interactions on enamel

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Abstract: OBJECTIVES This study aimed to analyze interactions between oral biofilms and a dental triethylene glycol dimethacrylate (TEGDMA)-based resin infiltration material on enamel. METHODS Demineralized enamel specimens (14 days, acidic buffer, pH 5.0) were either infiltrated with a commercial TEGDMA resin and subjected to a three-species biofilm (Streptococcus mutans UAB 159, Streptococcus oralis OMZ 607 and Actinomyces oris OMZ 745) (group 1), applied to the biofilm (group 2), or merely resin infiltrated (group 3). A control group received no treatment (4). Biofilm formation and metabolic activity of biofilms were measured for group (1) and (2) after 24h CFU and a resazurin assay. Resin biodegradation was measured for group (1) and (3) by high performance liquid chromatography (HPLC) coupled with mass spectrometry after 6 and 24h incubation. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) images were taken to study the biofilm and material's autofluorescence in groups (1-4) after 24h. RESULTS SEM and CLSM images showed reduced biofilm formation on resin-infiltrated specimens (group 1) compared to group 2, while no biofilm was detectable on groups 3 and 4. CFU data (log10 CFU per mL) of group 1 showed significantly reduced bacterial numbers ($p<0.05$) compared to group 2. However, HPLC analysis of TEGDMA leakage after 6h and 24h revealed no differences between group 1 and group 3. CONCLUSIONS The results of the current study indicate that freshly resin-infiltrated enamel surfaces show a biofilm reducing effect, while monomer leakage was not affected by bacterial presence. CLINICAL SIGNIFICANCE Resin infiltrated enamel surfaces are constantly exposed to the oral microflora. Yet, it is not known how biofilms interact with enamel-penetrated resins and if and to which extent accessory alignments in oral hygiene are needed.

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Oral Biofilm and Caries-Infiltrant Interactions on Enamel

Short title: Biofilm and Caries-Infiltrant Interactions

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Keywords: caries, enamel, HPLC, oral biofilm, resin, TEGDMA

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ABSTRACT

Objectives: This study aimed to analyze interactions between oral biofilms and a dental triethylene glycol dimethacrylate (TEGDMA)-based resin infiltration material on enamel.

Methods: Demineralized enamel specimens (14 days, acidic buffer, pH 5.0) were either infiltrated with a commercial TEGDMA resin and subjected to a three-species biofilm (*Streptococcus mutans* UAB 159*, Streptococcus oralis* OMZ 607 and *Actinomyces oris* OMZ 745) (group 1), applied to the biofilm (group 2), or merely resin infiltrated (group 3). A control group received no treatment (4). Biofilm formation and metabolic activity of biofilms were measured for group (1) and (2) after 24 h CFU and a resazurin assay. Resin biodegradation was measured for group (1) and (3) by high performance liquid chromatography (HPLC) coupled with mass spectrometry after 6 and 24 h incubation. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) images were taken to study the biofilm and material's autofluorescence in groups (1-4) after 24 h.

Results: SEM and CLSM images showed reduced biofilm formation on resin-infiltrated specimens (group 1) compared to group 2, while no biofilm was detectable on groups 3 and 4. CFU data ($\log_{10}$ CFU per mL) of group 1 showed significantly reduced bacterial numbers ($p < 0.05$) compared to group 2. However, HPLC analysis of TEGDMA leakage after 6 h and 24 h revealed no differences between group 1 and group 3.

Conclusions: The results of the current study indicate that freshly resin-infiltrated

enamel surfaces show a biofilm reducing effect, while monomer leakage was not affected by bacterial presence.

Clinical significance: Resin infiltrated enamel surfaces are constantly exposed to the oral microflora. Yet, it is not known how biofilms interact with enamel-penetrated resins and if and to which extent accessory alignments in oral hygiene are needed.

INTRODUCTION

New approaches for prevention and so-called non-invasive or micro-invasive treatment are increasingly studied to avoid restorative treatment [1]. Resin infiltration is a micro-invasive treatment option for demineralized, non-cavitated enamel, which is not expected to remineralize anymore. Further caries progression is suppressed by the penetration of low viscous and light curable resins into porous enamel [2]. Positive side effects were found in masking white spot lesions by modifying the refractive index of demineralized enamel [3]. Although several studies provided insight into the mechanical and chemical properties of resin-infiltrated enamel [4-6], only very little is known about the interaction of oral microflora with resin-infiltrated enamel.

It is known that biofilms develop on all orally exposed surfaces and consist of different cross-linked bacteria and extracellular polymeric substances [7]. Bacteria in biofilms show a higher pathogenicity compared to their planktonic counterparts [8]. External parameters, such as surface properties, nutrition supply and pH in the surrounding media were shown to be relevant for the biofilm formation and

composition [9-12]. Yet, especially the role of different surface characteristics on biofilm growth is controversially discussed [7]. Some studies revealed no differences of biofilm formation on varying surfaces as on different saliva-coated composites and glass ionomer cements [13]. In contrast, other studies found significantly different levels of bacterial adherence on different restorative materials, irrespective of the initial saliva coating [14].

The resin infiltrant mentioned above is mainly composed of triethylene glycol dimethacrylate (TEGDMA). Besides its high penetration capability and wettability [15], TEGDMA has been reported to influence growth patterns of certain bacterial strains and biomass formation [16]. However, the impact of TEGDMA on bacterial growth patterns was investigated only under very controlled *in vitro* conditions e.g. with regard to selective bacterial species, showing contradictory effects depending on the concentration of TEGDMA and the pH of the surrounding media [17, 18]. Leakage of TEGDMA monomers was only investigated in set-ups with cured plain resin material in molds. TEGDMA leakage of demineralized enamel specimens after resin infiltration and their interaction with complex biofilms was, to the authors' knowledge, not investigated yet. Thus, it is still unknown whether caries lesions infiltrated with TEGDMA are a preferential site for biofilm formation or if the resin infiltrant exhibits some antibacterial effects on multispecies biofilms as found in the oral cavity. Furthermore, it is still unknown, how the enamel mesh affects TEGDMA leakage after resin infiltration.

The aim of this study was to investigate the initial formation of oral biofilms on resin-infiltrated demineralized enamel surfaces in comparison to mere demineralized enamel and the TEGDMA leakage of resin-infiltrated enamel specimens with and

without biofilms. The null hypothesis was that the groups are not significantly different with regard to bacterial adhesion or TEGDMA leakage.

Keywords: caries, enamel, HPLC, oral biofilm, resin, TEGDMA

MATERIALS & METHODS

Specimen preparation and biofilm formation

Demineralized bovine enamel specimens (n = 48) were allocated into four groups: 1) resin-infiltrated enamel with biofilm, 2) enamel with biofilm, 3) resin-infiltrated enamel 4) no treatment (control). Enamel specimens were produced using bovine incisors. In brief, crowns were cut off from the roots and stored in a 0.1% thymol solution (VWR International, Dietikon, Switzerland) for no longer than 6 month. Cylindrical specimens (4 mm in diameter) were punched out from each crown and ground stepwise from 1200 to 4000 Fepa P (1200, 2400, 4000 grit, Water Proof silicon carbide Paper, Struers, Erkath, Germany).

Initial carious lesions with intact surface layer were created in all enamel specimens by the following demineralization procedure. Briefly, specimens were immersed for 14 days in an acidic buffer with traces of thymol at a pH of 5 and 37 °C [6, 19]. The solution was renewed every second day to maintain a constant pH. After demineralization, control specimens (n = 6) were cut in 100 µm slices and demineralization depth and intact surface layer were controlled using transverse microradiography. In this study, lesions of about 200 µm were used. Resin infiltration with Icon (DMG, Hamburg, Germany), a caries infiltrant system, was performed for specimens of group (1) and (3) according to the manufacturer's instructions. In brief,

15% hydrochloric acid was used to etch the surfaces for 2 min (Icon Etch, DMG) and removed by 30 s water rinsing and subsequent air-drying. Specimens were then rinsed with 99% ethanol (Icon Dry, DMG) for 30 s and air-dried. 0.75 µL resin (Icon Infiltrant, DMG) was applied for 3 min. Excess material was removed with a cotton roll prior to light-curing for 40 s. This was followed by a second application of 0.75 µL of resin for 60 s and gentle cotton roll application. Light curing followed again for 40 s (800 W/cm^2 bluephase, Ivoclar Vivadent, Schaan, Liechtenstein). Subsequently, specimens were fine grinded using a holder with an integrated spring and silicon carbide discs (4000 Fepa P) for 10 s to remove the oxygen inhibition layer under constant pressure. Specimens of all groups underwent gas sterilization for 16 h (Ethylene oxide, 3M^{TM} Steri-Gas^{TM} Cartridges, Healthcare, Rüschlikon, Switzerland) and were stored sterile in a moist chamber until further treatment.

For biofilm formation in groups (1) and (2), three-species biofilms were grown on specimens for 24 h. The bacterial strains (*Streptococcus mutans* OMZ 918, *Streptococcus oralis* OMZ 60, *Actinomyces oris* OMZ 745) were provided by the Institute for Oral Biology, Section for Oral Microbiology and General Immunology, University of Zurich, Zurich, Switzerland. Prior to bacterial incubation, specimens were immersed in diluted saliva supernatant to form a pellicle. One healthy subject donated fresh whole mouth saliva, which was used in all experiments. Donated non-stimulated saliva was centrifuged twice for 30 min (12'100 g). The supernatant was diluted 1:2 in 0.9% NaCl (Braun, Melsungen, Germany) and subsequently sterile filtrated (TPP syringe filters with 0.2 µm pores, Faust, Schaffhausen, Switzerland). For pellicle formation, specimens were incubated with gentle agitation in 800 µL diluted saliva supernatant for 4 h. Bacterial strains in a mixture of 30% diluted saliva

supernatant and 70% modified fluid universal medium (mFUM) [20, 21] were adjusted to an OD_{550} of 1 and mixed as inoculum. Pellicle-coated specimens were incubated during gentle agitation in 2 mL inoculum. Incubation was performed anaerobically in jars using gas-paks for 24 h at 37 °C (GENbox anaer and GENbag anaer, bioMérieux, Marcy l'Etoile, France). After 6 h, specimens were transferred to new wells with fresh medium and analyzed after 24 h incubation, whereas expended media (after 6 and 24 h incubation) were subjected to high performance liquid chromatography (HPLC).

HPLC

Analysis of uncured Icon Infiltrant (DMG) was carried out using HPLC on an Agilent 1100 LC/MS (Agilent Technologies, Basel, Switzerland). An Agilent column (ZORBAX Eclipse XDB-C8, 4.6 x 150 mm, 5 µm) with a 50/50 mixture of acetonitrile and water at a flowrate of 0.75 mL/min and a run time of 10 min was used. Identification of uncured Icon Infiltrant, namely TEGDMA, was performed with single ion detection at 309 m/z after diluting the experimental media (n = 12) with 50 vol% of a methanol-water (Millipore) mixture (80/20). To quantify the amount, the chromatogram peak area was compared with a calibration curve (linear range, R^2 = 0.998) of mere TEGDMA (95%). All chemicals for HPLC analysis were purchased from Sigma Aldrich (Buchs, Switzerland) and were of HPLC analytical grade, except otherwise stated.

Bacteria counts and metabolic activity

Plate counts were performed for specimens w/wo resin infiltration and subjected to

biofilm (groups 1 and 2, n = 8) after 24 h. Specimens were sonified in 1 mL 0.9% NaCl and vortexed. 50 µL of different bacterial dilutions (in 0.9% NaCl) were plated out on Columbia sheep blood agar plates (CSBA, bioMérieux, Geneve, Switzerland) and incubated under anaerobic conditions using gas-paks. Plate counting followed after 2 days of incubation using a light microscope with 10-fold magnification (Wild Stereoskop, Heerbrugg, Switzerland).

Bacterial metabolic activity of groups (1) and (2) was measured after 24 h (n = 10). Specimens were transferred into 96-well plates and incubated in 300 µL resazurin solution consisting of 10 vol% alamarBlue Cell Viability Assay Reagent (Life Technologies, Zug, Switzerland) and fresh media (30% saliva solution + 70% mFUM) under anaerobic conditions. Two wells were additionally filled with blank resazurin solution (without specimens for background detection) and one well with bacteria from the planktonic inocula. After 15 min, 200 µL of each solution was pipetted into new 96-well plates and fluorescence was measured in a spectrophotometer with plate-reader at 560 nm excitation / 585 nm emission at 37 °C (Spectramax M2, Molecular Devices, Bucher Biotec, Basel, Switzerland). Results were presented as relative fluorescence units (rfu) after background subtraction.

Bacteria imaging

Two specimens of each group were analyzed by scanning electron microscopy (SEM) after 24 h of incubation (SUPRA 50VP and Genesis, Carl Zeiss, Oberkochen, Germany). Briefly, specimens were washed with 0.9% NaCl solution and fixed for at least 24 h in 4% glutaraldehyde solution (in 0.1 M sodium potassium phosphate buffer, pH 7). A dehydration procedure followed [22] and subsequent critical point

drying was performed. Specimens were coated by gold sputtering for 60 s. Images were taken to show the detailed surface characteristics'.

Biofilms and resin-infiltrated surfaces were examined using confocal laser scanning microscopy (CLSM) after 24 h of incubation. Two specimens of each group were washed in 0.9% NaCl to remove loosely bound bacteria, fixed with 4% paraformaldehyde in the dark at room temperature for 60 min and washed again with 0.9% NaCl. Fluorescence staining was performed using Syto 59 (Life Technologies) to visualize all bacteria. The detailed staining procedure has been described elsewhere [23]. In brief, each specimen was incubated in 500 µL staining solution consisting of 5 µM Syto 59 for 15 min at room temperature in the dark. After staining, specimens were washed in phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA, USA) and mounted onto chamber slides (Fisher Scientific, Schwerte, Germany) using Mowiol 4-88 (Sigma Aldrich). Images were taken with a CLSM (SP5, Leica Microsystems, Heidelberg, Germany) using a 20x objective (numerical aperture: 1.25) and a helium laser (561 nm). Emission was detected with a photomultiplier between 630 – 660 nm. Three random areas of each specimen were examined with a z step size of 1 µm (512 x 512 pixels). Image processing was performed using Imaris Software 7.7.2 (Bitplane, Zurich, Switzerland).

Data presentation analysis

Leakage of TEGDMA monomers was compared between the resin-infiltrated specimens with biofilm (group 1) and without biofilm (group 3) after 6 h and after 24 h. Plate counts and relative fluorescence units were separately analyzed after 24 h between group (1) and group (2). All values were not normally distributed and

Wilcoxon/Kruskal-Wallis test was applied for non-parametrical analysis. JMP (version 10, SAS, Cary, NC, USA) was used for statistical analysis. The overall level of significance was set at $p \leq 0.05$.

RESULTS

HPLC analysis of resin-infiltrated specimens with biofilm coating (group 1) and without biofilm coating (group 3) revealed no significant differences in TEGDMA-leakage ($p > 0.05$). Leaked TEGDMA monomers (median) of group (1) resulted after 6 h in 0.040 x 10^{-3} µL per mm^2 and after 24 h in 0.110 x 10^{-3} µL per mm^2. Group (3) exhibited 0.027 x 10^{-3} µL per mm^2 after 6 h and 0.042 x 10^{-3} µL per mm^2 after 24 h (Figure 1). CFU results of bacteria on resin-infiltrated specimens showed significantly less bacterial colonization (group (1): 6.25 ± 0.26 $\log_{10}$ CFU/mL) than on demineralized enamel surfaces without resin infiltration (group (2): 6.60 ± 0.34 $\log_{10}$ CFU/mL) after 24 h (Table). There was no significant difference between the detected metabolic activity of group (1) 6.3 ± 2.7 x 10^3 rfu and group (2) 7.0 ± 3.2 x 10^3 rfu after 24 h (Table). SEM imaging of group (1) showed single islands and aggregates of bacterial biofilms as well as bacteria free regions exhibiting a slightly degraded infiltrated surface without any sign of demineralized enamel (Figure 2a). Images of resin-free surfaces with biofilms (group 2) revealed huge amounts of biofilm with peaks of bacterial islands and aggregates with rod-shaped and coccoid bacteria (Figure 2b). Group (3) images showed rough enamel surface regions with distinct enamel prisms and partially overhanging peaks of the infiltrant (Figure 2c). Demineralized surfaces of group (4) showed plain enamel areas with typical

demineralization pattern as exposed prism heads and accentuated demineralization of the interprismatic regions (Figure 2d). CLSM images of group (1) showed a deep red fluorescence of single bacterial aggregates (live and permeable) on a red fluorescing background, hampering the discrimination between bacteria and the surface. Yet, bacterial aggregates seemed to be surrounded by a less fluorescing yard (Figure 3a). Demineralized enamel specimens of group (2) showed biofilm-covered surfaces without red background fluorescence (Figure 3b). Resin-infiltrated enamel of group (3) showed red fluorescence at interprismatic gaps (Figure 3c). Group (4) revealed no fluorescence at all (Figure 3d).

DISCUSSION

Freshly resin-infiltrated enamel specimens reduced initial biofilm formation, while biofilms on infiltrated specimens revealed no effect on TEGDMA leakage. The reduction in plate counts was confirmed by SEM and CSLM images. However, metabolic activity of biofilm-coated resin-infiltrated specimens and mere biofilm specimens exhibited no significant differences in activity. Therefore, the null hypothesis was rejected for the effect of the bacterial adhesion but not for the TEGDMA leakage.

In order to perform this study adequately, two main challenges had to be solved. First, the penetration capacity of the resin infiltrant had to be standardized to the best possible extent. Second, monomer leakage should only be investigated in infiltrated enamel. Top layers of mere resin had to be avoided. The above described holding device for the curing light and dental air water spray contributed to a standardized

infiltration. A defined volume of 1.5 µL was used to infiltrate demineralized enamel. However, a calculation of the percental TEGDMA leakage was unfeasible due to the use of cotton rolls and the surface fine grinding after light curing. Yet, these steps were important to provide infiltrated specimens without an overhanging resin layer. SEM images of infiltrated specimens confirmed the resin infiltration within the enamel and CLSM images the interprismatic penetration within the demineralized enamel (Figures 2 & 3). Evaluations on the resin infiltration into enamel and on the demineralization depth were described using different CSLM approaches [24-26]. However, these approaches are based on sliced specimens and a calculation of the entire infiltrated volume remains barely practicable. Studies on the monomer elution of resin composite specimens revealed different results according to time, pH of the surrounding media and polymerization time and intensity. Different resin composite eluates such as the dental base monomers bisphenol A glycol dimethacrylate (BisGMA) or urethane dimethacrylate (UDMA), or the co-monomers ethylenglycol dimethacrylate (EGDMA), diethylenglycol dimethacrylate (DEGDMA) or TEGDMA were investigated under different conditions, using different media, storage time or polymerization periods up to 80 s [27-31]. Interestingly, a pronounced TEGDMA leakage was detected in most studies. However, long-term storage of specimens resulted in a decrease of TEGDMA leakage after 24 h, while other monomers remained at a high level for up to 28 d [29, 32, 33]. The present study investigated only the effect of freshly resin-infiltrated specimens within 24 h. Uncured TEGDMA monomers were found after 6 h with a slight increase in TEGDMA for 24 h. Bacterial adhesion did not seem to influence the elution of TEGDMA within this incubation period, although the elution was slightly wider distributed for biofilm exposed

specimens (Figure 1). TEGDMA elution, however, seemed to reveal slightly biofilm-inhibiting properties and resulted in lower plate counts. Other studies detected a growth-stimulating effect of TEGDMA on single bacterial strains, such as *Streptococcus sobrinus* and *Lactobacillus acidophilus* [17]. However, Takahashi et al. (2004) indicated, that growth-stimulating effects from previous studies might account for absorbance-based measurements and actually be caused by polymerized TEGDMA vesicular materials surrounding bacterial cells. It was concluded, that some bacteria initiate a polymerization of leached uncured TEGDMA monomers around their cells [16]. The vesicular-structured polymers could protect the bacterial cells and increase their resilience and also lead to more plaque accumulation due to increased surface roughness. Yet, monomers seem to interact with bacteria adhering to the surfaces in many different ways. Esterase activities of Streptococci species such as of *S. mutans* were shown to degrade monomers into several by-products [34]. Khalichi et al. (2004) investigated the effect of TEGDMA derived degradation products methacrylic acid (MA) and triethylene glycol (TEG). Growth-inhibiting or -stimulating effects were shown in a concentration and pH dependent manner. While TEG was found to stimulate the growth of *S. mutans* in low concentrations (0.5-10.0 mmol/L) and low pH (5.5), MA was reported to inhibit its growth at low and neutral pH. In the present study, fewer bacteria were found on infiltrated specimens compared to mere demineralized enamel. Therefore, further studies seem necessary to investigate the formation and effect of TEGDMA by-products on resin-infiltrated specimens.

CONCLUSION

This study presented for the first time the interaction of oral biofilms with resin-infiltrated enamel. There was no difference in monomer leakage if the biofilm was applied or not on the exposed surface. However, significantly less bacteria were detected on resin-infiltrated enamel specimens, indicating that these surfaces can inhibit bacterial growth, next to their restorative function.

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TABLE

Table. Bacterial numbers (Means ± SD, log_{10} CFU/mL) and metabolic activity (10^3 rfu) of biofilm-coated specimens with and without resin-infiltration after 24 h.

Group	CFU/mL (log_{10})	alamarBlue (x 10^3 rfu)
1	6.25 ± 0.26^{A}	6.3 ± 2.7^{a}
2	6.60 ± 0.34^{B}	7.0 ± 3.2^{a}

Quantification of biofilm-coated resin-infiltrated specimens (group 1) and biofilm on demineralized enamel (group 2) using CFU and alamarBlue assay.

Identical superscript letters within CFU or alamarBlue analysis' indicate that there was no significant difference (P <.05, Wilcoxon test for non-parametrical analysis) for this test between respective groups.

FIGURES

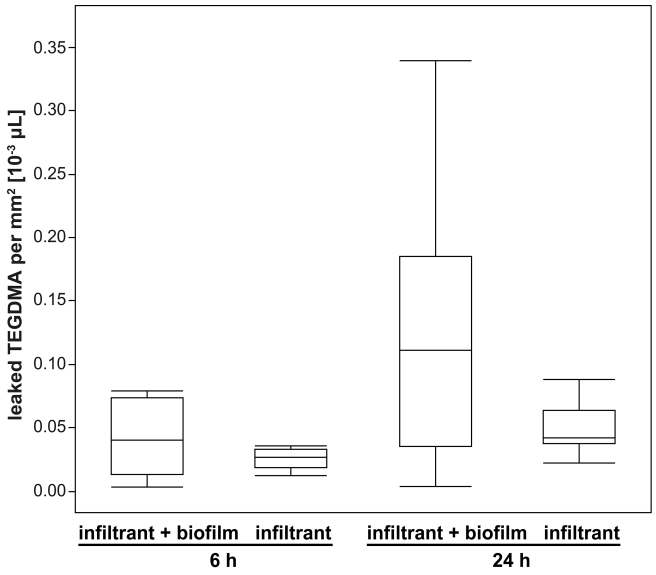

Figure 1. Uncured infiltrant, as determined by TEGDMA leakage (w/out biofilm exposition), recovered after 6 and 24 h incubation was measured by high performance liquid chromatography. No significant differences were shown between both groups. Resin-infiltrated specimens with biofilm (group 1) and without (group 3) are presented graphically.

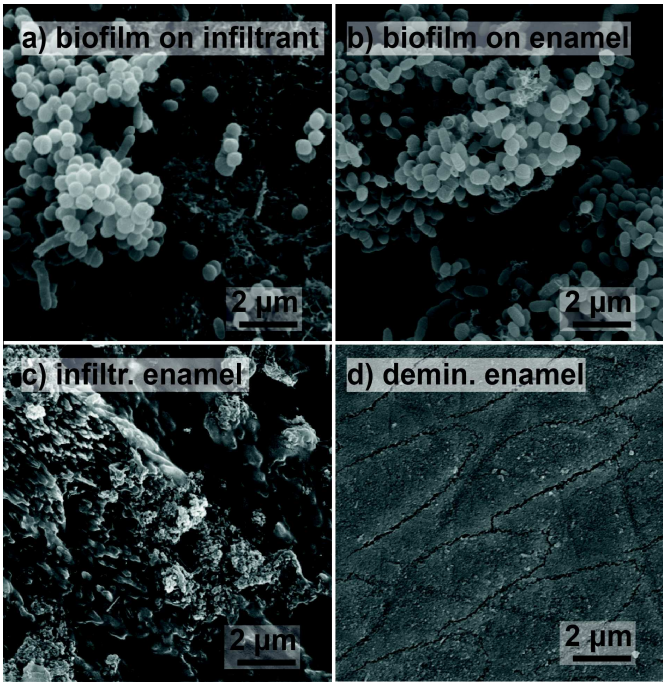

Figure 2. Scanning electron microscopy images of a) biofilm on resin-infiltrated enamel, b) biofilm on demineralized enamel, c) mere demineralized enamel after resin infiltration and d) demineralized enamel without biofilm.

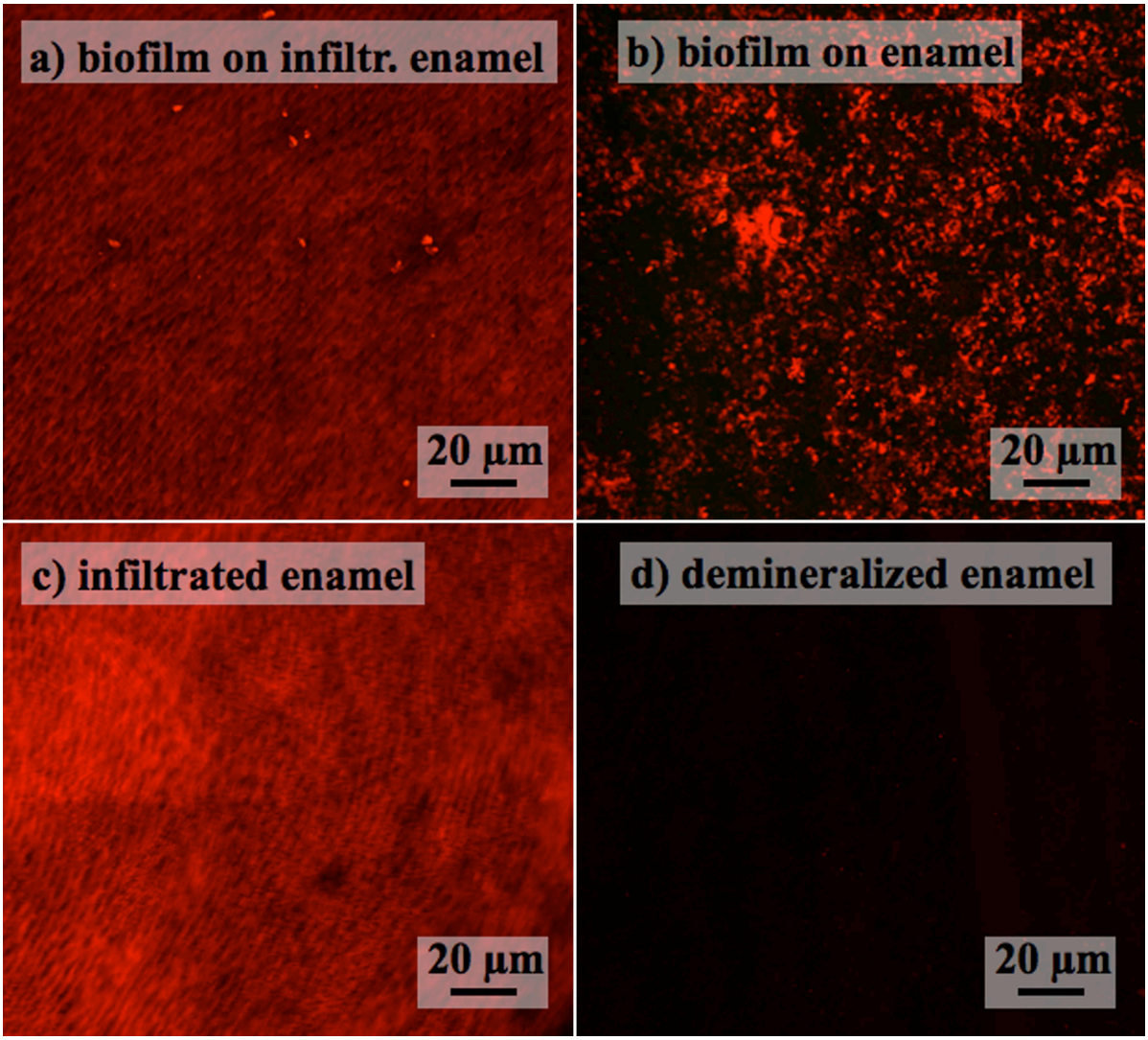

Figure 3. Confocal laser scanning microscopic images after staining with Syto 59 of a) biofilm on resin-infiltrated enamel, b) biofilm on demineralized enamel, c) mere demineralized enamel after resin infiltration and d) demineralized enamel without biofilm.